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Porcine Teschovirus, Sapelovirus and Enterovirus in Swiss Pigs: Investigation of Prevalences and Disease Association by Multiplex RT-PCR

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CONTENTS

ZUSAMMENFASSUNG

Porzine Teschoviren (PTV), porzine Sapeloviren (PSV-A) und porzine Enteroviren (EV-G) sind enterische Viren, die hauptsächlich im Zusammenhang mit gastrointestinalen, neurologischen, respiratorischen und Reproduktionserkrankungen bei Schweinen beschrieben, aber auch in asymptomatischen Haus- und Wildschweinen nachgewiesen werden. Für die meisten Serotypen gibt es bisher keinen Beweis eines kausalen Zusammenhangs zwischen Infektion und Erkrankung.

In dieser Studie wurde ein multiplex RT-PCR Protokoll angepasst, um den Nachweis der Viren in Gehirn-, Kot- und Plazenta-/Abortproben gesunder und erkrankter Schweizer Schweine zu verbessern.

In Plazenta-/Abortproben sowie in Hirnproben gesunder Schweine wurde keines der Viren nachgewiesen. Im Hirngewebe erkrankter Schweine wurde eine geringe Anzahl Proben positiv getestet (PSV-A in 6.2% und EV-G in 4.9%). PTV wurde in keiner der Hirnproben nachgewiesen. Somit gab es keine Hinweise auf die meldepflichtige Teschener Krankheit.

In den Kotproben konnten hingegen hohe Prävalenzen gefunden werden. Bei gesunden Tieren wurde PTV in 47.4%, PSV-A in 51.3% und EV-G in 69.7% nachgewiesen. Erkrankte Schweine zeigten Prävalenzen von 54.3% für PTV, 64.2% für PSV-A und 67.9% für EV-G. Absetzferkel und Mastschweine hatten höhere Prävalenzen als Saugferkel und Mutterschweine. Co-Infektionen mit drei Viren wurden am häufigsten detektiert. Mittels statistischer Analysen konnte kein Zusammenhang zwischen Virusnachweis und Krankheit nachgewiesen werden.

Key words: Enterovirus G; Porzin; Prävalenz; RT-PCR; Sapelovirus; Teschovirus.

ABSTRACT

Porcine Teschovirus (PTV), porcine Sapelovirus (PSV-A) and porcine Enterovirus (EV-G) are enteric viruses that have been associated with several diseases, primarily gastrointestinal, neurologic, respiratory and reproductive disorders but also with subclinical infections in pigs and wild boars. However, for most serotypes proof for a causal relationship of virus infection with clinical signs is still lacking.

In this study, a previously described multiplex RT-PCR protocol was adapted to improve detection of the viruses in brain, fecal and placental/abortion samples of healthy and diseased Swiss pigs. None of the 3 viruses were detected in placental/abortion samples nor in brain samples of healthy pigs. In brain tissue of diseased pigs, a small number of PSV-A (6.2%) and EV-G (4.9%) positive samples were detected. Notably, with all the brain samples tested negative for PTV there was no evidence for the reportable Teschen disease.

In contrast, the viruses were highly prevalent in fecal samples of healthy as well as diseased pigs. In healthy animals PTV was detected in 47.4%, PSV-A in 51.3%, and EV-G in 69.7% of the samples, while in diseased animals prevalences accounted to 54.3%, 64.2% and 67.9%, respectively. Prevalences in fecal samples were higher among weaned and fattening pigs compared to suckling piglets and sows, and co-infections with all 3 viruses were most frequently detected. Statistical analysis yielded no evidence for an association of virus detection and disease.

Key words: Enterovirus G; Porcine; Prevalence; RT-PCR; Sapelovirus; Teschovirus.

SUBMITTED MANUSCRIPT

Porcine Teschovirus, Sapelovirus and Enterovirus in Swiss Pigs: Investigation of Prevalences and Disease Association by Multiplex RT-PCR

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Running title: Prevalences and Disease Association of Porcine Tescho-, Sapelo- and Enteroviruses in Swiss Pigs.

Abstract. Porcine Teschovirus (PTV), porcine Sapelovirus (PSV-A) and porcine Enterovirus (EV-G) are enteric viruses infecting pigs and wild boars worldwide. The viruses have been associated with several diseases, primarily gastrointestinal, neurologic, respiratory and reproductive disorders but also with subclinical infections. However, for most serotypes proof of a causal relationship between virus infection and clinical signs is still lacking. In the present study a previously described multiplex RT-PCR protocol was adapted, in order to improve detection of the 3 viruses in brain, fecal and placental/abortion samples of healthy and diseased Swiss pigs. The 3 viruses were detected neither in placental/abortion samples nor in brain samples of healthy pigs. In brain tissue of diseased pigs, a small number of PSV-A (6.2%) and EV-G (4.9%) positive samples were detected. Notably, with all the brain samples tested negative for PTV there was no evidence for the reportable Teschen disease. In contrast, all the 3 viruses were detected at high prevalences in fecal samples of both healthy and diseased pigs. In healthy animals PTV was detected in 47.4%, PSV-A in 51.3%, and EV-G in 69.7% of the samples, while in diseased animals prevalences accounted to 54.3%, 64.2% and 67.9%, respectively. Virus prevalences in fecal samples were higher among weaned and fattening pigs compared to suckling piglets and sows, and co-infections with all 3 viruses were most frequently detected. Based on clinical and pathological data, statistical analysis yielded no evidence for an association of virus detection and disease.

Key words: Enterovirus G; Porcine; Prevalence; RT-PCR; Sapelovirus; Teschovirus.

Introduction

Enteric viruses of the species Teschovirus A and B, Sapelovirus A and Enterovirus G (EV-G) all belong to the *Picornaviridae* family (https://ictv.global/taxonomy/) and are known to infect pigs and wild boars. Teschovirus A includes 13 serotypes, designated as porcine Teschovirus 1 to 13 (PTV 1-13).[43] Recently, several novel putative serotypes of PTV were identified and a new species, Teschovirus B, was described.[27, 41] For Sapelovirus A, only a single serotype is described, named porcine Sapelovirus 1 (PSV-1), while 17 subtypes of EV-G are known to infect pigs (G1 – G4, G6, G8 – G19, https://ictv.global/taxonomy/).[43] The viruses are distributed worldwide and have been reported in America,[2, 3, 10, 32] Europe,[5, 6, 30] Africa,[13] and Asia.[8, 18, 22] Infections with PTV and PSV-A have been reported in association with neurologic disorders, i.e. paresis or paralysis[3, 9, 19, 32, 33, 37, 39], diarrhea[19, 21, 45], respiratory disorders[19, 21, 45], and reproductive failure.[22] However, subclinical infections have also been described.[5, 30, 39] Highly virulent strains of PTV-1 evidentially cause a severe neurologic disease known as Teschen disease.[1] Less virulent strains of PTV-1 as well as other PTV serotypes can lead to mild neurologic disorders, known as Talfan disease or benign enzootic paresis.[1] EV-G has mostly been reported in association with subclinical infection[30, 35, 40] but has also been isolated from aphthae like skin lesions.[16] Reported prevalences for the 3 enteric virus species primarily derive from fecal samples and show considerable differences, ranging from 2.8 – 46.4% for PTV[10, 30, 36, 42], 17.5 – 72.8% for PSV-A[4, 7, 10, 30], and 40.0 – 56.5% for EV-G.[10, 30, 36] Despite frequent detection in diseased animals, proof for an association of virus infection with clinical signs is still lacking for most serotypes.

Until recently, there was no diagnostic test available in Switzerland for detection of any of the 3 virus species. Especially for Teschen disease, which is classified as a reportable epizootic disease in Switzerland, a diagnostic assay was needed. Consequently, only little is known about the occurrence of these viruses in Swiss pigs. Therefore, the aims of the present study were (i) the establishment and validation of a multiplex RT-PCR protocol for simultaneous detection of PTV, PSV-A and EV-G in porcine samples, (ii) the determination of the prevalences of PTV, PSV-A and EV-G by applying the newly established RT-PCR protocol on fecal, brain and placental/abortion samples from healthy and diseased Swiss pigs and, (iii) based on the clinical and pathological data of the animals, the investigation of a possible association with disease, especially gastrointestinal, neurologic and/or reproductive disorders.

Materials and methods

Sample material

Clinical specimens investigated included brain, fecal and placental/abortion samples from both healthy and diseased pigs (Tab. 1). Thirty-one brain samples from healthy fattening pigs (A1) were collected by official veterinarians at the municipal slaughterhouse in Zurich in autumn 2019. Seventy-six fecal (B1) and 11 placental samples (C1) from clinically healthy pigs were collected by veterinarians of the Swiss Pig Health Service between summer and winter 2019. Brain (A2) and fecal (B2) samples from 81 pigs sent to the Institute of Veterinary Pathology at the University of Zurich between 2014 and 2015 were also included. Eighty-three placental/abortion samples from sows showing reproductive disorders (C2), including abortions, mummies, stillborn and weak born piglets, had been taken within the framework of a previous project.[12] Unfortunately, from the available data we were unable to trace which fetal organs were contained in the samples tested in our study. All samples were stored at -20°C until processing.

Table 1: Overview of clinical specimens analyzed. A total of 363 samples from 282 animals were investigated and classified into 6 groups.

Number of samples	Sample material	Animals	Group
31	Brain	Healthy fattening pigs	A1
81 & 81	Brain & Feces	Pigs with various diseases	A2 & B2
76	Feces	Healthy pigs	B1
11	Placenta	Sows without reproductive disorders	C1
83	Placenta/ abortion samples	Sows with reproductive disorders	C2
363		282	

Sample preparation and RNA extraction

Twenty-five mg of brain or placental/abortion samples were mixed with 560 µL of AVL buffer (QIAamp Viral RNA Mini Kit; Qiagen, Hilden, Germany) and disrupted in a TissueLyser II (Qiagen) at 30 Hz for 20 sec using a 5 mm stainless steel bead (Qiagen). The tubes were then centrifuged at 6000 x g for 1 min. For the fecal samples, 1 g or 1 mL of feces was mixed with 4 mL of phosphate-buffered saline (PBS) and two 2 mm glass beads (Faust, Schaffhausen, Switzerland) and vortexed thoroughly. Of this mixture, 1 mL was centrifuged at 6000 x g for 1 min. RNA was extracted from the supernatant (Qiagen) according to the manufacturer's instructions. The extracted RNA was stored at –20°C until further use.

PTV, PSV-A and EV-G multiplex RT-PCR

For amplification of PTV-, PSV-A- and EV-G-RNA, a nested multiplex RT-PCR protocol published by Zell et al. (2000)[44] was modified: in order to reduce the contamination risk, RT-PCR was performed as single-run assay using only the inner primer pairs (Tab. 2). For better sensitivity, the cycle number was increased from 35 in the original protocol to 40. Since the original PSV-A primers (pev-8c and pev-8d) according to sequence alignments did not fit well on the 3D polymerase gene region of recently detected Swiss PSV-A strains (Fig. 1; C. I. Rickli, personal communication, 2020),[31] they were replaced by PSV-A primers (pev-8g and pev-8h) published by Krumbholz et al. (2003).[17] Just like the PTV and EV-G primers,[44] they target the more conserved 5' UTR region of the PSV-A genome and aligned perfectly to the genome of Swiss PSV-A strains (Fig. 2, C. I. Rickli, personal communication, 2020).[31] Analogous alignments showed that PTV and EV-G primers from Zell et al. (2000)[44] fit nicely on Swiss strains (data not shown, C. I. Rickli, personal communication, 2020),[31] therefore no adjustment had to be made for these primers.

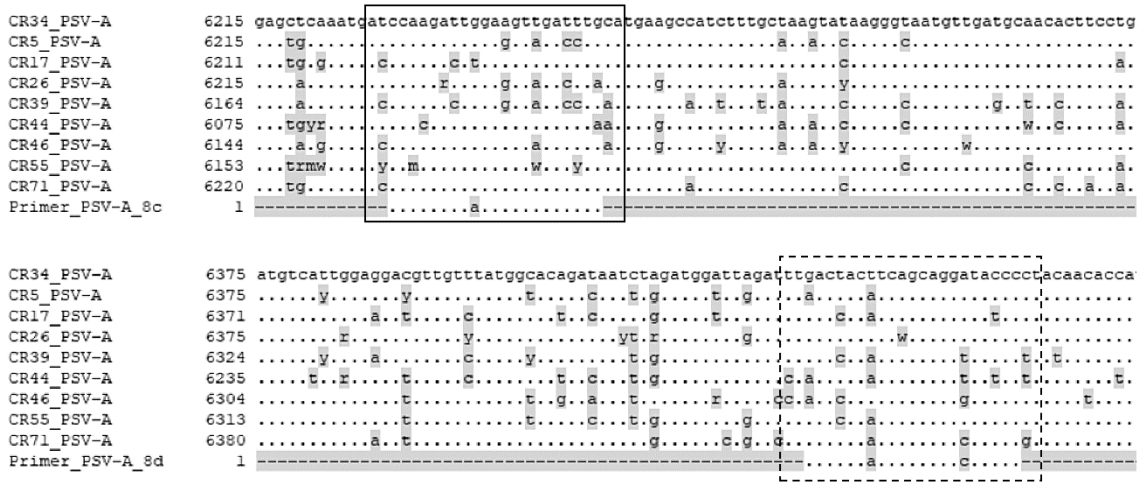

Figure 1: Alignment of PSV-A primers according to Zell et al. (2000) to Swiss PSV-A strains in the 3D pol region. Binding regions of the forward (pev-8c) and the reverse primer (pev-8d) are highlighted by boxes with a continuous line and a dashed line, respectively.

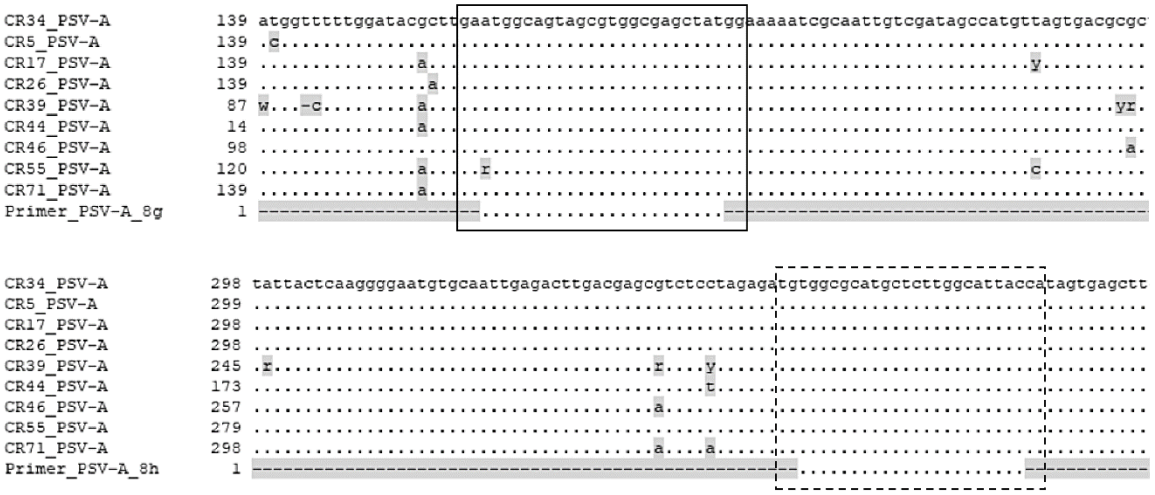

Figure 2: Alignment of PSV-A primers according to Krumbholz et al. (2003) to Swiss PSV-A strains in the 5' UTR region. Binding regions of the forward (pev-8g) and the reverse primer (pev-8h) are highlighted by boxes with a continuous line and a dashed line, respectively.

Henceforth, we refer to the single-run multiplex RT-PCR using the PSV-A primers by Zell et al. (2000) as original protocol (OP) and to the single-run RT-PCR using the PSV-A primers published by Krumbholz et al. (2003) as novel protocol (NP).

Table 2: Primer sequences used for single-run multiplex RT-PCR.

Virus	Gene region	Sequence	Fragment size (bp)	Primer name	Reference
PTV	5' UTR	5'-TGAAAGACCTGCTCTGGCGCGAG-3'	158	pev-1c	Zell et al., 2000
		5'-GCTGGTGGGCCCCAGAGAAATCTC-3'		pev-1d	
PSV-A	3D pol	5'-CCAAGATTAGAAGTTGATTTG-3'	221	pev-8c	Zell et al., 2000 (OP)
		5'-GGGTAGCCTGCTGATGTAGTC-3'		pev-8d	
	5' UTR	5'-ATGGCAGTAGCGTGGCGAGCTAT-3'	212	pev-8g	Krumbholz et al., 2003 (NP)
		5'-GTAATGCCAAGAGCATGCGCCA-3'		pev-8h	
EV-G	5' UTR	5'-CAAGCACTTCTGTTTCCCCGG-3'	313	pev-9c	Zell et al., 2000
		5'-GTTAGGATTAGCCGCATTCA-3'		pev-9d	

3D pol=RNA-dependent RNA polymerase 3D gene region, 5' UTR=5' untranslated region, EV-G=enterovirus G, NP=novel RT-PCR protocol, OP=original RT-PCR protocol, PSV-A=porcine sapelovirus A, PTV=porcine teschovirus.

The OneStep RT-PCR Kit (Qiagen) was used to set up the RT-PCR reaction. The mix had a final volume of 25 µL and included 5 µL OneStep RT-PCR buffer (5x), 400 µM of each dNTP, 600 nM of each primer (pev-1c/-1d, pev-8g/-8h, and pev-9c/-9d), 1 µL OneStep RT-PCR enzyme mix, 0.1 µL RNase inhibitor (RNasin; Promega, Madison, WI) and a top-up of nuclease-free water to which 5 µL extracted RNA were added. Samples of

all groups were tested undiluted and in 1:10 dilution to exclude PCR inhibition. Thermal cycling was performed on a FlexCycler^2 (Analytik Jena, Jena, Germany) and consisted of a reverse transcription step at 50°C for 30 min, followed by 95°C for 15 min, 40 cycles of 50 sec at 94°C, 50 sec at 55°C and 1 min at 72°C, and a final extension at 72°C for 5 min. After cycling, the samples were cooled down to 4°C. The amplified products were analyzed on a 2% agarose gel at 80 V for ~ 70 min. Bands of the expected sizes (Tab. 2) were excised and DNA was extracted using the QIAquick Gel Extraction Kit (Qiagen). DNA concentration was measured on a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Per 100 bp of expected product, 18 ng of DNA were mixed with 3 µL of the respective forward primer (10 µM) and topped-up with nuclease-free water to a final volume of 15 µL. The samples were sent to Microsynth (Microsynth, Balgach, Switzerland) for sequencing. Sequences were analyzed with NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). In case of an unclear sequencing result, the sample was resequenced using the corresponding reverse primer (10 µM) or the RT-PCR was repeated. Since unspecific amplifications were observed, only samples that showed a band of expected size after gel electrophoresis and that yielded sequences with highest identity to the expected virus were considered positive.

p12S real-time RT-PCR

Amplification of the porcine 12S rRNA (p12S) housekeeping gene served as internal control, in order to confirm successful RNA extraction. The reaction mix had a final volume of 10 µL and contained 5 µL RT-PCR (2x) mix (TaqMan RNA-to-CT 1 step kit; Applied Biosystems, Thermo Fisher Scientific, Waltham, MA), 500 nM of each primer (Tab. 3), 250 nM p12S probe (Tab. 3), 0.25 µL RT enzyme mix (40x; Applied Biosystems) and a top-up of nuclease-free water to which 1 µL extracted RNA (10- or 100-fold diluted in nuclease-free water) were added. Real-time RT-PCR was run on a QuantStudio™ 7 Flex Real-Time PCR Instrument (Applied Biosystems). Thermal cycling included a reverse transcription step at 48°C for 15 min, followed by 95°C for 10 min and 45 cycles at 95°C for 15 s and 60°C for 1 min.

Table 3: Primer and probe sequences used for the p12S real-time RT-PCR.

Primer/ probe	Sequence
p12S Forward	5’ – CCA CCT AGA GGA GCC TGT TCT ATA A – 3’
p12S Reverse	5’ – GGC GGT ATA TAG GCT GAA TTG G – 3’
p12S Probe	FAM – CGA TAA ACC CCG ATA GAC CTT ACC AAC CC – TAMRA

p12S=porcine 12S rRNA.

Clinical and pathological data

Brain samples of healthy pigs (A1) were examined histologically in order to exclude microscopic alterations. For diseased animals of group A2/B2 a post-mortem examination had been conducted by the Institute of Veterinary Pathology at the University of Zurich. In most cases necropsy included histological investigation and further testing for specific pathogens was undertaken. Clinical data, especially whether the animals had suffered from diarrhea, neurologic or reproductive disorders were obtained from the referring veterinarians. Based on the clinical and pathological data, animals of group A2/B2 were categorized into five main syndrome groups: (i) gastrointestinal tract (GIT) disorders; (ii) systemic disorders (SD); (iii) musculoskeletal disorders; (iv) neurologic disorders; and (v) indeterminable disorders. The GIT and SD groups were further divided into sub-groups: (i) GIT, diarrhea; (ii) GIT, hemorrhagic bowel syndrome (HBS), (iii) GIT, unknown clinical signs/history; (vi) SD, with neurologic signs and with diarrhea; (v) SD, without neurologic signs but with diarrhea; (vi) SD, with neurologic signs but without diarrhea; (vii) SD, without neurologic sign and without diarrhea; (viii) SD, without neurologic signs, without diarrhea but with abortion. Post-mortem examinations had also been conducted for group C2, and samples were tested for specific pathogens.[12] Samples of groups B1 and A2/B2 were divided into four age categories: (i) suckling piglets less than 5 weeks of age; (ii) weaned pigs with 5 – 10 weeks of age; (iii) fattening pigs with 11 – 27 weeks of age; and (iv) adult sows.

Data analysis

Prevalences within all samples, animals, groups A1 – C2, and age categories were calculated. Two different kinds of prevalences are described: “overall prevalences” indicate how frequently the individual viruses were detected while “mono-/co-infections” describe the frequencies of all the possible infection statuses including negative, mono-, double- (PTV and PSV-A, PTV and EV-G, or PSV-A and EV-G) and triple- (PTV, PSV-A, and EV-G) infections. For statistical analyses, IBM SPSS Statistics for Windows, version 25.0 (IBM Corp., Armonk, N.Y., USA) was used. A *p*-value less than .05 was considered statistically significant. Fishers-exact test was used to check for differences in the prevalences between the different groups, between the different age categories within the same group, and between healthy and diseased animals within the same sample material and age category. In order to ascertain if the presence of viruses may be associated with a certain clinical picture, virus prevalences of diseased pigs, categorized into the above named main syndrome groups and sub-groups (A2/B2), were compared with those from healthy pigs (B1) using binary logistic regression analysis. This test was further used to check for an association between

etiological diagnosis within the different syndromes (clear vs. uncertain/unknown etiology) and virus detection in order to ascertain whether disease in cases with an uncertain etiology could be explained by the presence of one or more of the 3 viruses.

Results

Comparison between OP and NP

Samples of groups A1, A2, B2 and C2 were tested with both the old (OP) and the new protocol (NP). When tested with the OP, only 7.4% (6/81) of the samples in group B2 were found to be positive for PSV-A, while all samples of groups A1, A2 and C2 tested negative. In contrast, using the NP, 6.2% (5/81) of the samples in group A2 and 64.2% (52/81) in group B2 were found PSV-A positive, while the samples of groups A1 and C2 were confirmed negative. As a consequence, samples of groups B1 and C1 were only tested with the NP. Accordingly, the following results refer to data obtained using the NP.

Prevalences of PTV, PSV-A and EV-G in Switzerland

The study included 363 samples of 282 animals that were tested for the presence of the 3 viruses. PTV was detected in 28.4% (80/282), PSV-A in 32.3% (91/282), and EV-G in 38.7% (109/282) of animals tested. In 56.0% (158/282) of the animals no virus was detected. On sample level, PTV was found in 22.0% (80/363), PSV-A in 26.4% (96/363), and EV-G in 30.9% (112/363) of the samples. The remaining samples (63.6%; 231/363) tested negative (Tab. 4).

Table 4: Overview of the overall virus prevalences within the different study groups on sample basis (n=363).

Overall infections	A1 (n=31)	A2 (n=81)	B1 (n=76)	B2 (n=81)	C1 (n=11)	C2 (n=83)	Total (n=363)
PTV	-	-	47.4%	54.3%	-	-	22.0%
PSV-A	-	6.2%	51.3%	64.2%	-	-	26.4%
EV-G	-	4.9%	69.7%	67.9%	-	-	30.9%
Negative	100%	88.9%	19.7%	23.5%	100%	100%	63.6%

EV-G=enterovirus G, PSV-A=porcine sapelovirus A, PTV=porcine teschovirus.

In brain samples, viral RNA was only detected in samples of diseased pigs (A2), whereas in fecal samples viruses were detected in both healthy and diseased animals (B1 and B2; Tab. 4). Samples of group A2 tested PSV-A positive in 6.2% (5/81) and EV-G positive in 4.9% (4/81). The remaining 88.9% (72/81) tested negative. Notably, no PTV nor any co-infections were detected in those brain samples (Tab. 5). Samples of group B1 tested PTV positive in 47.4% (36/76), PSV-A positive in 51.3% (39/76), and EV-G positive in 69.7% (53/76). In 19.7% (15/76) no virus was detected. Co-infections with all 3 viruses were

detected most frequently (35.5%, Tab. 5). In group B2, PTV was detected in 54.3% (44/81), PSV-A in 64.2% (52/81), and EV-G in 67.9% (55/81) of the samples. In 23.5% (19/81) of the samples none of the 3 viruses were detected. As for group B1, co-infections with all 3 viruses were the most frequent (44.4%, 36/81; Tab. 5). Except for one sow, where EV-G was detected in the brain but not in the fecal sample, all animals that tested PSV-A or EV-G positive in the brain samples (A2) also had a positive result in the corresponding fecal sample (B2). Viral RNA was not detected in placental/abortion material (C1 and C2).

Table 5: Overview of mono- and co-infection prevalences within the different study groups on sample basis (n=363).

Mono-/ Co-infections	A1 (n=31)	A2 (n=81)	B1 (n=76)	B2 (n=81)	C1 (n=11)	C2 (n=83)	Total (n=363)
PTV	-	-	1.3%	2.5%	-	-	0.8%
PSV-A	-	6.2%	9.2%	3.7%	-	-	4.1%
EV-G	-	4.9%	17.1%	4.9%	-	-	5.8%
PTV & PSV-A	-	-	-	2.5%	-	-	0.6%
PTV & EV-G	-	-	10.5%	4.9%	-	-	3.3%
PSV-A & EV-G	-	-	6.6%	13.6%	-	-	4.4%
PTV, PSV-A & EV-G	-	-	35.5%	44.4%	-	-	17.4%
Negative	100%	88.9%	19.7%	23.5%	100%	100%	63.6%

EV-G=enterovirus G, PSV-A=porcine sapelovirus A, PTV=porcine teschovirus.

Comparison of prevalences between the different age categories

For group A2, no significant difference in the overall prevalences was observed between the different age categories (PTV p = 1.000., PSV-A p = 1.000, EV-G p = .103, negative p = .200; Fig. 3).

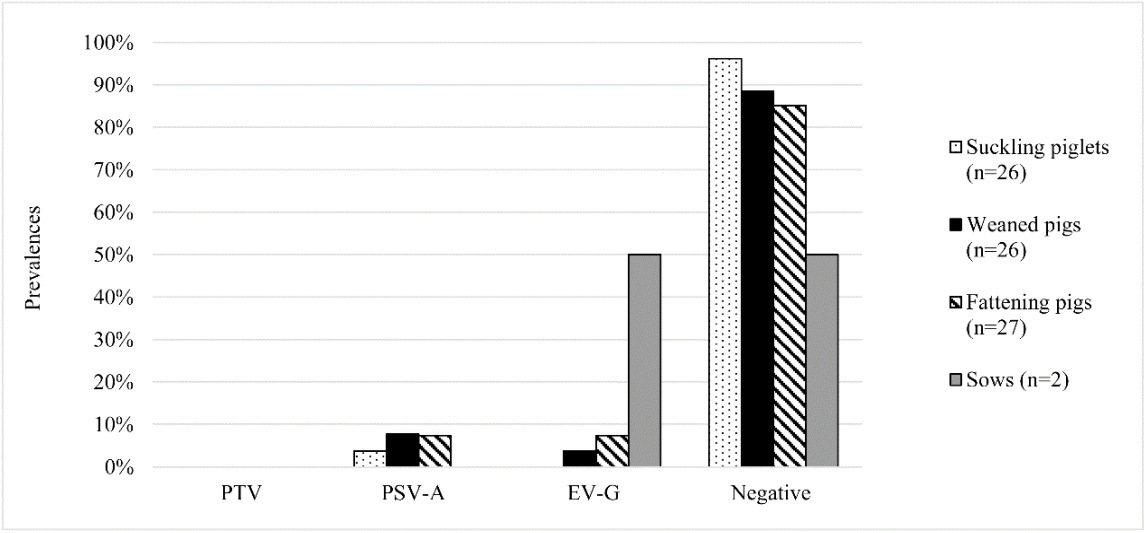

Figure 3: Overall prevalences within the four age categories of group A2.

When comparing the overall prevalences between the four age categories of group B1, significant differences for all 3 viruses were observed between suckling piglets and weaned pigs (PTV $p < .001$, PSV-A $p < .001$, EV-G $p < .001$, negative $p = .008$) as well as suckling piglets and fattening pigs (PTV $p = .002$, PSV-A $p = .042$, EV-G $p = .001$, negative $p = .020$; Fig. 4). The same applies for the differences between sows and weaned pigs (PTV $p < .001$, PSV-A $p < .001$, EV-G $p < .001$, negative $p = .001$) as well as sows and fattening pigs (PTV $p < .001$, PSV-A $p = .022$, EV-G $p < .001$, negative $p = .002$; Fig. 4).

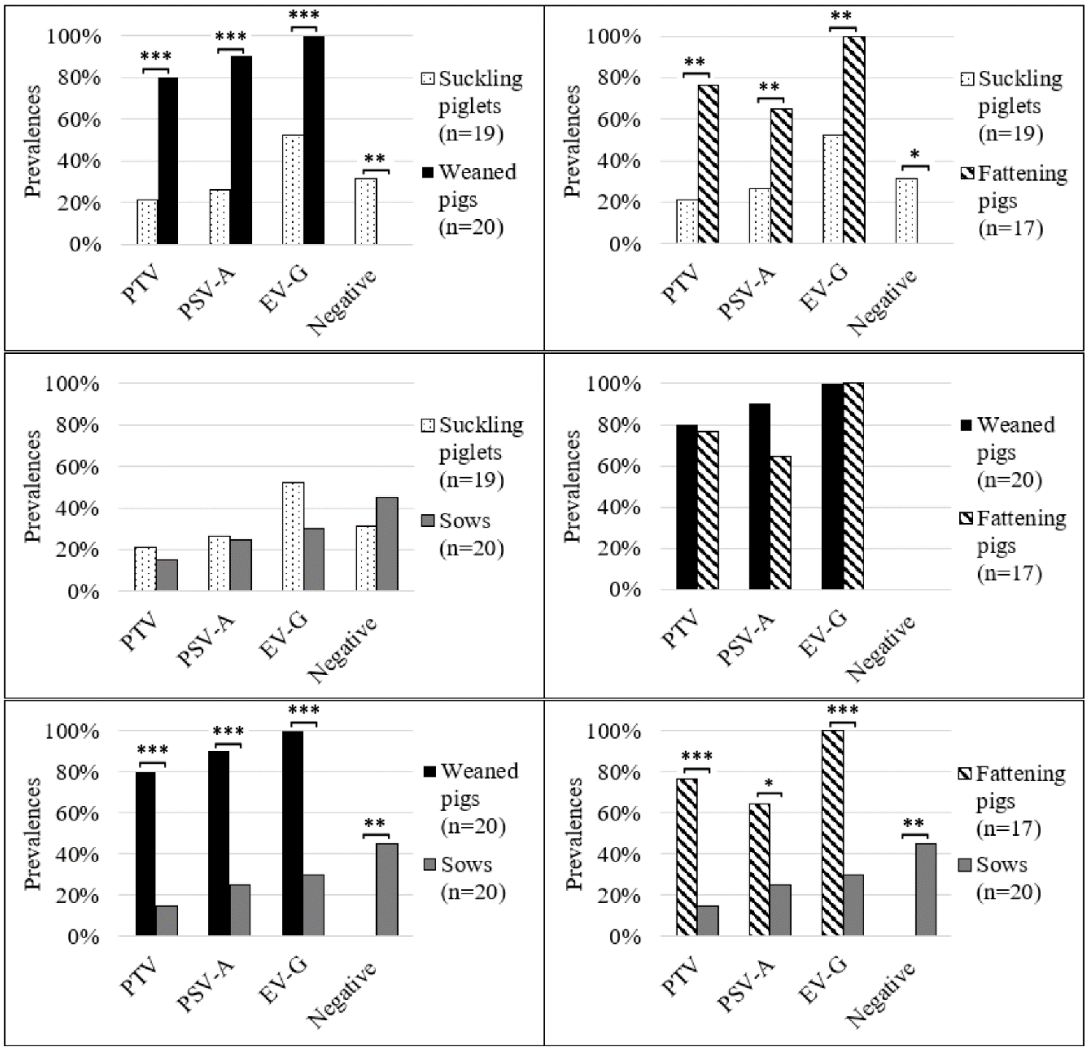

*Figure 4: Overall prevalences within the four age categories of group B1, significance level stated as follows: * p= <.05, ** p= <.01, and *** p= <.001.*

In all cases, weaned and fattening pigs were more frequently infected than suckling piglets and sows. No significant differences in the virus prevalences were found between suckling piglets and sows (PTV $p = .695$, PSV-A $p = 1.000$, EV-G $p = .200$, negative $p = .514$) nor between weaned and fattening pigs (PTV $p = 1.000$, PSV-A $p = .109$, EV-G $p = 1.000$, negative $p = 1.000$; Fig. 4).

Comparison of mono-/co-infections between the four age categories of group B1 yielded similar results (Fig. 5). Again, no significant differences were seen between suckling piglets and sows (p = .882) nor weaned and fattening pigs (p = .270). The only statistically significant differences were observed for triple-infections, with weaned and fattening pigs being significantly more often infected with all 3 viruses than suckling piglets (p < .001 and p = .001, respectively) and sows (p < .001 and p < .001, respectively).

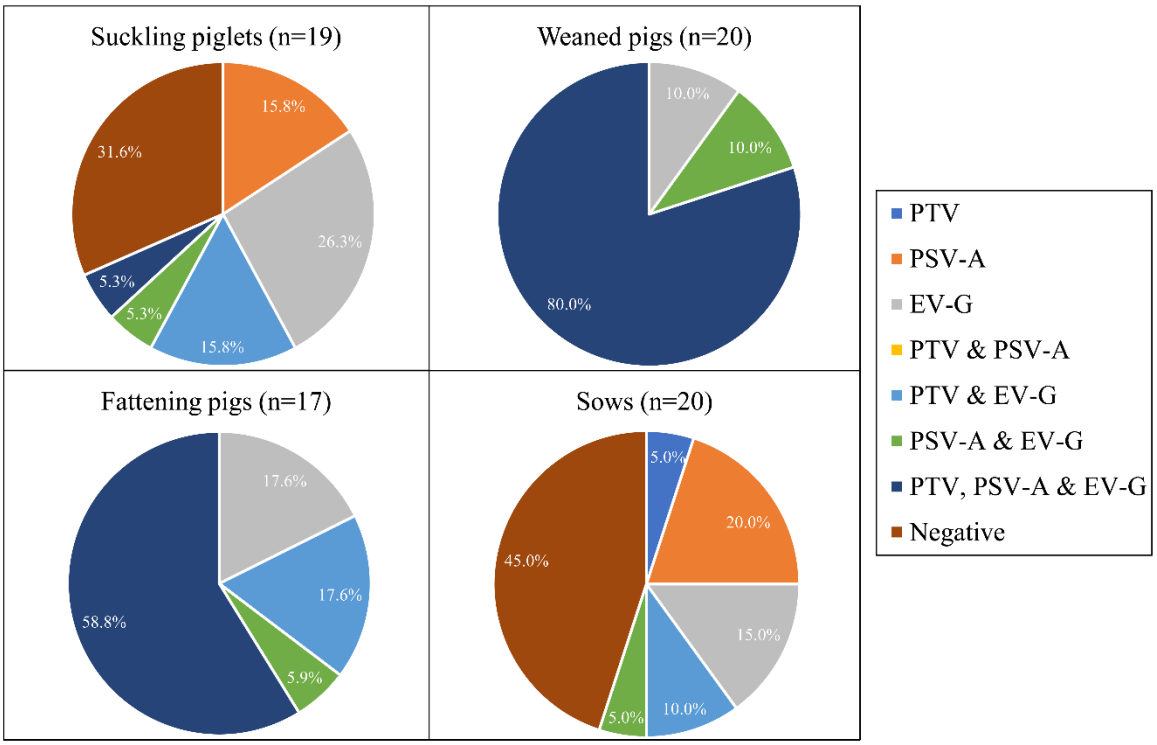

Figure 5: Prevalences of mono-/co-infections within the four age categories of group B1.

Samples of group B2 showed no significant differences in the overall prevalences between suckling piglets and sows (PTV p = 1.000, PSV-A p = 1.000, EV-G p = .492, negative p = .484; Fig. 6) and always significantly higher prevalences in fattening pigs than suckling piglets (PTV p < .001, PSV-A p < .001, EV-G p = .004). The remaining prevalences varied considerably between the different age categories (Fig. 6). Weaned pigs were significantly more frequently infected with PSV-A and EV-G than suckling piglets (p < .001 and p = .045, respectively), and suckling piglets significantly more often tested negative compared to weaned pigs (p = .006). PTV was found significantly more often in fattening than weaned pigs (p = .003). Weaned pigs showed significantly more infections with PSV-A (p = .026) and significantly fewer negative results than sows (p = .026). PTV and EV-G were significantly more often found in fattening pigs than sows (p = .025 and p = .037, respectively) while sows were more likely to have a negative test result (p = .007). For the

remaining prevalences no significant differences were detected between the different age categories ($p > .05$; Fig. 6).

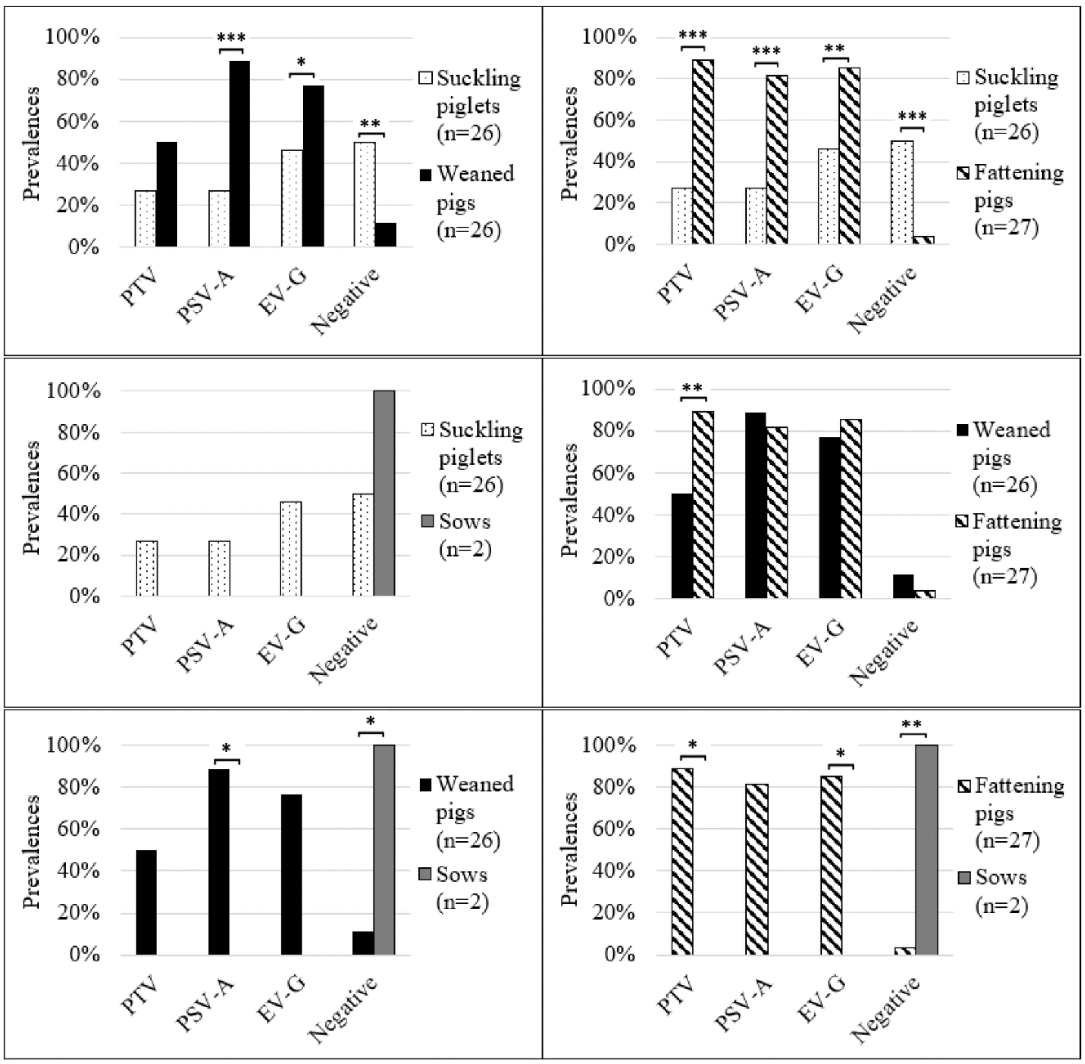

*Figure 6: Overall prevalences within the four age categories of group B2, significance level stated as follows: * p= <.05, ** p= <.01, and *** p= <.001.*

Looking at mono-/co-infections, only 2 combinations were found to be statistically significant. Triple-infections occurred significantly more often in fattening pigs than in suckling piglets ($p < .001$; Fig. 7) and weaned pigs were significantly more likely to be double-infected with PSV-A and EV-G than fattening pigs ($p = .011$, Fig. 7).

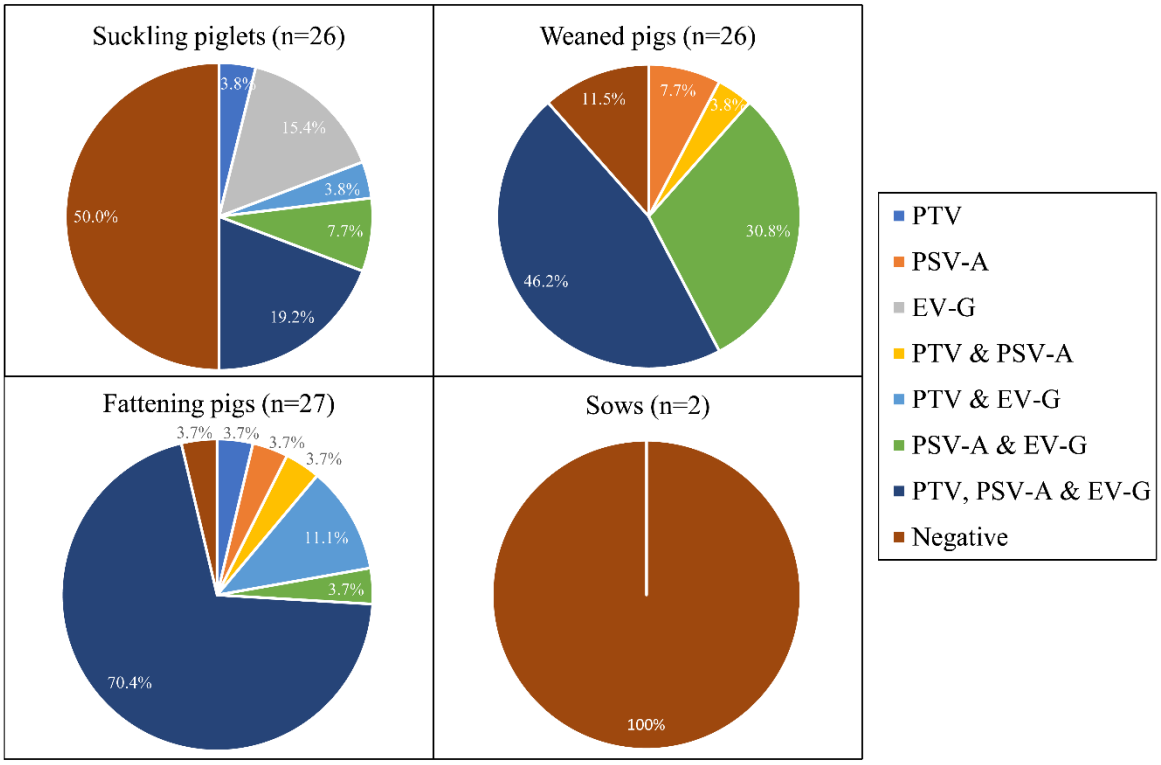

Figure 7: Prevalences of mono-/co-infections within the four age categories of group B2.

Comparison of prevalences between healthy and diseased animals

When comparing healthy and diseased animals (A1 and A2, B1 and B2 as well as C1 and C2), no significant differences ($p > .05$) in the overall prevalences were detected between those groups regardless of age. Within the different age categories, again no significant differences were found between healthy and diseased pigs, except for fecal samples of healthy weaned pigs (B1), that showed a significantly higher EV-G prevalence than the ones of diseased pigs (B2) of the same age ($p = .029$; Fig. 8). Considering mono-/co-infections, no significant differences in the prevalences between healthy and diseased suckling piglets, fattening pigs, and sows were observed ($p > .05$). However, among weaned pigs, healthy animals (B1) were more likely to have a co-infection with all 3 viruses compared to diseased pigs (B2) of the same age ($p = .032$).

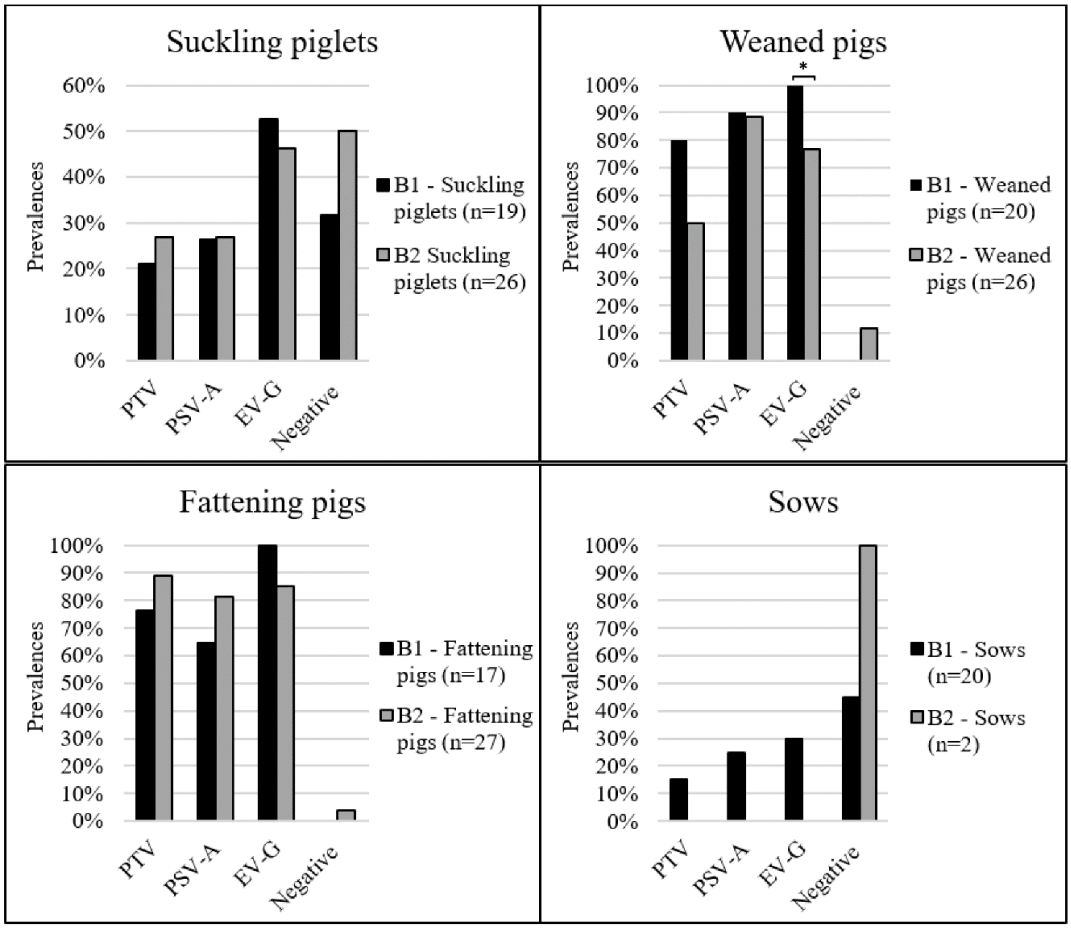

Figure 8: Comparison of the overall prevalences between fecal samples of healthy (B1) and diseased (B2) pigs of each age category.

Assessment of a possible association of virus detection with disease

Binary logistic regression analysis was only performed on fecal samples (B1 and B2), since in the other groups the viruses were either not detected (A1, C1 and C2) or detected at a very low rates (A2). Virus prevalences within the different main syndrome groups and sub-groups of diseased pigs (B2) were compared with those of healthy pigs (B1; Tab. 6). The binary logistic regression model did not find any association ($p > .05$) for the main syndromes and presence of virus. Within the different sub-groups of GIT and SD disorders no statistically significant results ($p > .05$) were found except for the syndrome "SD, without neurologic disorder and without diarrhea". The logistic regression model was statistically significant when only including EV-G ($\chi^2(2) = 7.049$, $p = .029$), indicating an association with this clinical picture and the presence of EV-G (Wald(1) = 5.173, $p = .023$), with Nagelkerke R^2 values of 18.5%. For the same syndrome, the regression model also showed statistical significance ($\chi2(1) = 3.875$, p = .049) when co-infection with PSV-A and EV-G was included (Wald(1) = 3.910, p = .048), with Nagelkerke R^2 values 10.4%. However, when including all the 3 viruses into the model, no significant differences ($p > .05$) were no longer observed.

Table 6: Overall virus prevalences within the main syndrome groups and sub-groups of group B2.

	GIT; overall (n=33)	GIT; Diarrhea (n=25)	GIT; HBS (n=7)	GIT; Unknown (n=1)	SD; overall (n=36)	SD; neurologic disease Yes, Diarrhea Yes (n=2)	SD; neurologic disease No, Diarrhea Yes (n=4)	SD; neurologic disease Yes, Diarrhea No (n=22)	SD; neurologic disease No, Diarrhea No (n=7)	SD; neurologic disease No, Diarrhea No, Abortion Yes (n=1)	Musculoskeletal diseases (n=5)	Neurologic diseases (n=4)	Indeterminable disorders (n=3)
PTV	60.6%	56%	85.7%	-	44.4%	-	50%	50%	42.9%	-	80%	50%	66.7%
PSV-A	66.7%	68%	71.4%	-	55.6%	50%	75%	59.1%	42.9%	-	60%	100%	100%
EV-G	81.8%	80%	85.7%	100%	55.6%	-	75%	68.2%	28.6%	-	60%	100%	33.3%
Negative	18.2%	20%	14.3%	-	33.3%	50%	25%	2.7%	42.9%	100%	20%	-	-

EV-G=enterovirus G, GIT=gastrointestinal tract, PSV-A=porcine sapelovirus A, PTV=porcine teschovirus, SD=systemic disease.

For samples of group A2/B2, according to pathological data a clear etiology had been stated in 60.5% (49/81) of the cases whereas in 39.5% (32/81) no etiological diagnosis (uncertain/unknown) could be determined by the pathologists, including all cases of HBS. When comparing virus occurrence of cases with an uncertain/unknown etiological diagnosis and cases with a clear etiological diagnosis within the different syndrome groups and sub-groups with binary logistic regression analysis, no significant differences were found ($p > .05$).

Discussion

Until recently, there was no diagnostic test available in Switzerland for the detection of PTV, PSV-A or EV-G. Especially for PTV the establishment of a diagnostic assay was essential, since highly virulent strains of PTV-1 are known to cause the reportable Teschen disease. Even more, by modifying and optimizing a previously published multiplex RT-PCR protocol for detection of PTV, PSV-A and EV-G,[44] the new assay supports the detection of pathogenic PTV and thereby helps to close a diagnostic gap. It further allows to gain more insight into the occurrence of the 3 viruses in Switzerland and into a possible association with certain clinical signs, especially gastrointestinal, neurologic or reproductive disorders. However, since the multiplex RT-PCR does not allow clear discrimination of the different serotypes, additional diagnostic tools such as amplification with specific primers[44] or next generation sequencing (NGS) should be employed in case of a positive result, especially for

PTV positive brain samples in order to specify whether the infecting virus is a highly virulent PTV-1 strain causing Teschen disease.

The NP using PSV-A primers according to Krumbholz et al. (2003) showed a much higher sensitivity than the OP using the PSV-A primers published by Zell et al. (2000). More precisely, with the NP 56.8% more samples of group B2 were found PSV-A positive compared to the OP, and 6.2% more in group A2. These findings demonstrate the significance that sequence information on circulating strains in a specific population can have for primer design. Furthermore, it highlights the importance of periodical re-evaluation of already established PCR protocols with respect to currently circulating strains, especially for rapidly evolving and highly diverse viruses like the picornaviruses.[23, 27, 34, 41, 43] In some cases, gel electrophoresis repeatedly yielded visible bands at the expected size but sequencing generated no result at all or the obtained sequences did not correspond to the expected virus. This might be due to a very low viral load in the sample or due to unspecific amplifications. Since these samples were considered negative, the reported prevalences in this study might be slightly underestimated.

While all brain samples of healthy pigs (A1) tested negative, brain samples of diseased pigs (A2) tested positive for PSV-A and EV-G, albeit at very low rates (PSV-A = 6.2%; EV-G = 4.9%). Both viruses have previously been detected in brain samples of animals suffering from neurologic disease. In general, previously described prevalences in brain samples were higher than those found in our study. However, direct comparison of prevalences is difficult, since data from previous studies usually only refer to testing of diseased animals in the course of an outbreak, while healthy animals were not tested. In one study neurologic tissue from pigs with neurologic signs and typical virus-induced histologic lesions was tested for the three viruses[3] using an RT-PCR protocol previously described.[44] Single-infection with PSV-A was found in 31.6% (6/19), double-infection with PTV and PSV-A in 21.1% (4/19), double-infection with PSV-A and EV-G in 15.8% (3/19), and triple-infection in 5.3% (1/19) of the samples.[3] In an outbreak with respiratory distress, diarrhea, ataxia, paralysis and death in pigs in China,[19] all 17 organ pools that included brain samples as well as feces, serum, liver, spleen, lung and intestine, tested positive for PSV-A by RT-PCR.[29] The brain samples have not been tested separately and therefore no final statement can be made concerning virus presence in this tissue. Experimental infection of pigs with a PSV-A isolate caused clinical signs similar to those reported in the outbreak and supported re-isolation of the virus from fecal and lung samples. Additional to clinical signs also gross and histological changes were evident in the brain samples.[19] In the UK, an outbreak with neurologic signs, including front or hind leg weakness that progressed to generalized

weakness and lateral recumbency and sometimes even to death was described.[33] Four pigs were sent in for testing and all four pigs were found PSV-A positive in brain or spinal cord samples by pan-virus microarray.[33] Compared to previous reports, no co-infections in the brain samples were detected in our study. Notably, despite frequent detection of PTV in the feces, no PTV was detected in any of the brain samples. Bearing in mind that highly virulent PTV-1 would also be detectable in the brain after enteric infection, there was no evidence for the reportable Teschen disease. We therefore conclude that the PTV strains circulating in Swiss pigs are nonpathogenic or of low virulence and restricted to enteric infection, as has been described in other countries.[1, 24] However, as mentioned above, in case of positive brain samples, further analyses would be required to reach conclusion concerning the infecting serotype and its virulence.

In the fecal samples, all the 3 viruses were frequently detected in both healthy (B1) and diseased animals (B2). As for the brain samples, comparison of prevalences in fecal samples with previous reports is difficult and needs to be interpreted with caution, since different studies included animals with different clinical signs and different age categories. For group B1, PTV was found in 47.4% in our study and literature reports prevalences of healthy pigs between 24.7 – 46.9%.[10, 30, 42] Group B2 had a PTV prevalence of 54.3% whereas prevalences from previous reports range from 8.3% up to 52.2%.[10, 30, 42] Hence, both groups had slightly higher prevalences of PTV than previously reported. Prevalences of PSV-A in group B1 was 51.3%, prevalences previously reported in healthy animals range from 2.8% to 87.5%.[10, 13, 30, 36] Group B2 had a PSV-A prevalence of 64.2%, compared to 8.7 – 69.7% previously reported in diseased pigs.[10, 13, 30] Findings of our PSV-A prevalences are therefore in the range of already reported prevalences. For EV-G, healthy pigs of group B1 showed a prevalence of 69.7%. Previously reported prevalences range between 35.3 – 56.5%.[10, 30, 36] Group B2 had an EV-G prevalence of 67.9%, while previous studies reported prevalences between 43.5 – 51.2% in diseased pigs.[10, 30]

We also compared the prevalence data obtained from the four different age categories with those described in the literature. However, some of these studies did not differentiate between healthy and diseased animals, used different age categories, or did not include all age categories. Therefore, comparison of the here described prevalences and those reported previously was done regardless of health status for the different age categories. For both group B1 and B2, PTV prevalences for suckling piglets, weaned as well as fattening pigs were within those previously reported (Tab. 7).

Table 7: Overview of the overall PTV prevalences within the four age categories of groups B1 and B2 as well as previously reported prevalences.

PTV	B1	B2	Literature	References
Suckling piglets	21.1%	26.9%	6.7 – 27.1%	10, 30, 42
Weaned pigs	80.0%	50.0%	23.1 – 96%	
Fattening pigs	76.5%	88.9%	33.3 – 91.3%	
Sows	15.0%	-	10%	

PTV=porcine teschovirus.

Sows of group B1 showed higher prevalences than previously reported, however, only one study was found for this age category. On the other hand, sows of group B2 tested negative for PTV. However, with only 2 animals tested, these results cannot be considered representative. PSV-A prevalences in suckling piglets, weaned pigs, healthy fattening pigs, and diseased sows were within the reported prevalences (Tab. 8).

Table 8: Overview of the overall PSV-A prevalences within the four age categories of groups B1 and B2 as well as previously reported prevalences.

PSV-A	B1	B2	Literature	References
Suckling piglets	26.3%	26.9%	0 – 71.1%	4, 10, 13, 30
Weaned pigs	90.0%	88.5%	38.9 – 96.0%	
Fattening pigs	64.7%	81.5%	50.0 – 69.5%	
Sows	25.0%	-	0 – 12.5%	

PSV-A=porcine sapelovirus A.

In contrast, PSV-A prevalences in diseased fattening pigs and healthy sows were clearly higher than previously reported (Tab. 8). As demonstrated above, it is most probable that the adaption of the old RT-PCR protocol, i.e. the PSV-A primers, considerably contributed to these higher prevalences. With the exceptions of diseased suckling pigs and weaned pigs as well as sows of both groups, the EV-G prevalences reported here were consistently higher than previously observed (Tab. 9).

Table 9: Overview of the overall EV-G prevalences within the four age categories of groups B1 and B2 as well as previously reported prevalences.

EV-G	B1	B2	Literature	References
Suckling piglets	52.6%	46.2%	27.3 – 47.1%	5, 10, 30, 36
Weaned pigs	100%	76.9%	0 – 92%	
Fattening pigs	100%	85.2%	0 – 80.4%	
Sows	30%	-	20 – 31.3%	

EV-G=enterovirus G.

Our findings confirm the results of a recent report investigating the fecal virome of Swiss piglets by metagenomic analysis that described PTV and PSV-A to lie within the previously reported prevalences (32% and 50%, respectively) and also found a clearly higher prevalence for EV-G (63%, C. I. Rickli, personal communication, 2020).[31] In conclusion, these results indicate that EV-G is more widely distributed in Switzerland than in other countries, leading to a higher rate of EV-G infected pigs.

When looking into mono-/co-infections in the different age groups, triple-infections in weaned and fattening pigs of both groups (B1 and B2) were the most common finding, whereas samples of suckling piglets and sows most commonly yielded negative results. In the present study conducted in Switzerland, virus negative samples were also the most frequently detected in Swiss piglets (26%).[31] Previous studies found that suckling piglets are the least likely to be infected with all 3 viruses[13, 42] and that in the groups of weaned and fattening pigs triple-infections were most common.[30] These results could be confirmed in the present study. The increasing virus prevalence with age may be explained by the presence of protective maternal antibodies in suckling piglets and because sows in general show less virus infections, most probably due to protective immunity, leading to a lower infection pressure for the piglets. In contrast, weaned and fattening pigs are more prone to viral infections due to stressful or immunocompromising situations such as the decline of maternal antibody titer after weaning, moving into new premises or overcrowding.

With exception of one sow which was EV-G positive only in the brain, all other animals of group A2/B2 tested positive for either PSV-A or EV-G in the brain and the fecal sample. The reason for this result may be due to a very low virus load in the feces, PCR inhibition or contamination of the brain sample during necropsy or sample processing.

All placental/abortion samples (C1 and C2) tested negative for the 3 viruses. Samples of group C2 had been collected previously for a study on infectious reproductive disorders in Swiss pigs.[12] According to the authors, 44 out of 286 samples were randomly tested for PTV, PSV-A and EV-G by an already published RT-PCR.[44] PTV was found in 2.3% (1/44) and EV-G was found in 9.1% (4/44) of the samples, while no PSV-A was detected.[12] Unfortunately, we could not trace whether the 83 samples tested in the present study were represented in this earlier testing. In any case, the reported prevalences could not be confirmed in the present study. Suboptimal sample quality and RNA degradation over the years cannot be fully excluded. However, detection of a housekeeping gene in every sample clearly argues against RNA degradation. In China, PTV was detected in an outbreak of reproductive failure, including abortions, stillbirths and neonatal deaths.[22] Using a PCR previously described,[44] pooled samples of heart, liver, spleen, lungs, and intestine from the

fetuses/neonates tested PTV positive in 78.3% (54/69). Testing for other pathogens was negative, except for one organ pool sample where porcine Circovirus 2 (PCV-2) was detected.[22] Other studies investigating the virus prevalences of PTV, PSV-A and EV-G in sows with reproductive disorders are lacking. As for the brain samples comparison of prevalences in placental/abortion samples is difficult, since data from previous studies most often refer to testing of diseased animals in the course of an outbreak, while healthy animals were not tested.

Statistical analyses did not find any association for the different syndromes and virus detection, except for one syndrome (SD, without neurologic sign and without diarrhea), where the logistic regression model was statistically significant when including EV-G only or EV-G and PSV-A. However, when including all the 3 viruses into the model, no significant differences were no longer found, indicating that these few significant findings with only quite weak associations may be due to confounders. Also, according to statistical analysis cases with an unclear etiological diagnosis could not be explained by detection of one or more of the 3 viruses. In conclusion, the present study did not find any evidence for association between disease - especially gastrointestinal, neurologic or reproductive disorders - and virus detection. However, from the 9 cases where brain samples were found positive for either PSV-A or EV-G, in 5 cases no etiological diagnosis had been stated by the pathologists. Even though disease association was not statistically substantiated, it cannot be fully excluded that PSV-A or EV-G at least partially contributed to the observed clinical signs/syndromes.

Like most other studies already reported, our study did not find an association between diarrhea and PTV, PSV-A or EV-G detection in fecal samples[13, 30] or paraplegia and PSV-A detection in rectal as well as nasal swabs.[4] Notably, our results confirm findings of a recent study performing metagenomic analysis on fecal samples of Swiss piglets, where no significant association of PTV or PSV-A with diarrhea was found (C. I. Rickli, personal communication, 2020).[31] However, in the same study EV-G was detected significantly more often in piglets without diarrhea and a possible association with health was concluded.[31] Similar results were reported in a study where higher infection rates of PTV were observed in healthy pigs compared to diarrheic pigs.[41] Also in our study EV-G as well as triple-infections were significantly more often detected in healthy weaned pigs (B1) than in diseased pigs of the same age (B2). However, dilution of the virus in diarrheic feces below the detection limit cannot be ruled out in these cases.

These results are contradictory to a study that reported higher prevalences for all the 3 viruses in diarrheic pigs compared to healthy ones.[10] Our results also disagree with

results from experimental infections where neurologic disorders such as paresis or paralysis in the hind limbs,[11, 19, 25, 38, 40] diarrhea,[15, 19, 21, 25, 45] respiratory disorders[19, 45] or reproductive disorders, i.e. mummification, and fetal death,[14] were reported after inoculation with PTV, EV-G or PSV-A.

Possible explanations for the contradictory results and reasons why some infected animals show clinical signs while others do not may include different levels of virulence in different strains or serotypes,[14, 25] or co-infections with multiple serotypes, strains or other enteric pathogenic agents modifying the virulence or the host immune.[26, 28] In fact, the use of different strains and different numbers of cell culture passages are known to influence results of experimental infections.[11]

Indeed, results of the present study suggest co-infections with several serotypes/strains in some samples. Viral sequences of animals of group A2/B2 which tested positive for PSV-A or EV-G in both brain and fecal sample were aligned. While some alignments showed no difference in nucleotides between the brain and the fecal sample, indicating that the strain detected in the feces was the same that caused viremia and thereby spread to the brain, other sequences showed up to 11 nucleotides difference, indicating that the respective animal was infected with several different virus strains or serotypes, or mutations/recombinations of the strain occurred in the intestines over time.[20, 34] Since only short sequences of a highly conserved region were compared, no conclusions concerning the infecting strains or serotypes can be made. Also, in this context metagenomic analysis would be a valuable tool to detect co-infections with several viruses and simultaneously provide more insight into viral genomes and virus diversity.

Although the viruses have often been described in the context of respiratory disease, no lung samples were screened in this study but would clearly represent an interesting tissue for similar investigations. The assay established in this work allows now to routinely test a wide variety of pig tissue in Switzerland for the 3 viruses and will therefore contribute to our understanding of their role in health and disease.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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